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Fyn binding protein, Fyb, interacts with mammalian actin binding protein, mAbp1

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Abstract The immune cell specific protein Fyn-T binding protein (Fyb) has been identified as a target of the *Yersinia* antiphagocytic effector *Yersinia* outer protein H (YopH), but its role in macrophages is unknown. By using Fyb domains as bait to screen a mouse lymphoma cDNA library, we identified a novel interaction partner, mammalian actin binding protein 1 (mAbp1). We show that mAbp1 binds the Fyb N-terminal via its C-terminally located src homology 3 domain. The interaction between Fyb and mAbp1 is detected in macrophage lysates and the proteins co-localize with F-actin in the leading edge. Hence, mAbp1 is likely to constitute a downstream effector of Fyb involved in F-actin dynamics.

Keywords: Fyn-T binding protein; Mammalian actin binding protein 1; *Yersinia* outer protein H; F-actin; Vasodilator-stimulated phosphoprotein; src homology 3 domain

1. Introduction

Fyn-T binding protein (Fyb, also denoted SLAP-130 or ADAP) has been identified as a target of the *Yersinia* antiphagocytic factor, *Yersinia* outer protein H (YopH), in macrophages [1]. The protein tyrosine phosphatase (PTPase) YopH, is an essential virulence factor of pathogenic *Yersinia* species that invades lymphatic tissue and evades the primary immune defence [2]. When *Yersinia* interacts with target cells, YopH together with other Yop effectors are delivered into host cells via a type III secretion/translocation mechanism [3]. Macrophages, which constitute the first line of defence in lymphoid tissues, are considered as the major target cells for the antiphagocytic effect of *Yersinia*. YopH is one of the first effectors that enter into the cell, and its PTPase activity is required for phagocytic blockage and for *Yersinia* virulence in mice [4–6].

Phagocytosis is an actin-dependent process mediated by different receptors and the blocking by YopH includes phagocytosis mediated by integrins as well as Fc receptors [7]. Non-opsonized *Yersinia* binds to β1-integrins of host cells via the surface located protein, invasin [8]. This binding initiates signalling to the cytoskeleton that results in actin reorganization, which in the absence of YopH allows engulfment of the surface attached bacteria. When YopH is present and injected into the cells, this PTPase rapidly dephosphorylates certain tyrosine-phosphorylated proteins and this is associated with impaired phagocytosis [9,10]. In macrophages, the substrates of YopH are p130 Crk associated substrate (Cas), SKAP-55 homology protein (SKAP-HOM) and Fyb [1,11–13], also known as SLP-76 associated protein of 130 kDa (SLAP-130) [14] or adhesion and degranulation promoting adaptor protein (ADAP) [15].

The finding of Fyb as a target of YopH in macrophages is especially intriguing since expression of this adapter protein is restricted to immune cells. Fyb is expressed in T cells, mast cells, monocytes, platelets, but not B cells [16]. There are two Fyb isoforms of 120 and 130 kDa that differ because of a 46 amino acid insert in the C-terminus of the 130 kDa form [17]. Fyb is an adaptor protein that contains several regions with potential to mediate protein-protein interactions; these include several proline-rich motifs in the N-terminal half of the protein and a C-terminal src homology 3 (SH3)-like domain. In addition, Fyb also has two putative nuclear localization sequences and multiple tyrosine containing motifs. Two YDDV motifs have been shown to be tyrosine-phosphorylated by Fyn kinase, which is important for the binding to the SH2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) [14,17]. Fyb also contains a FPPPP motif, which has been reported to bind the Ena/VASP homology 1 (EVH1) domain of enabled/vasodilator-stimulated phosphoprotein (Ena/VASP), a family of actin binding and regulating proteins [18–20].

Most studies of Fyb have been focused on T cells, which is the cell type where Fyb was initially found. Several reports have suggested a role for Fyb in integrin-associated actin organization where Fyb influences the avidity of both β2 and β1 integrins [15,21–23]. Fyb −/− T cells fail to enhance integrin-dependent adhesion but T cell receptor (TCR) induced actin polymerization is normal [24,25]. In agreement with this, Fyb has been found to co-localize with F-actin in membrane ruffles, adhesion plaques/podosomes and phagocytic cups [15,26,27]. The identification of Fyb as a ligand for the EVH1 domain of the actin-binding Ena/VASP proteins [18]

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Abbreviations: YopH, *Yersinia* outer protein H; mAbp1, mammalian actin binding protein 1; SH3, src homology 3; Fyb, Fyn-T binding protein; PTPase, protein tyrosine phosphatase; Cas, p130 Crk associated substrate; SKAP-HOM, SKAP-55 homology protein; SLAP-130, SLP-76 associated protein of 130 kDa; ADAP, adhesion and degranulation promoting adaptor protein; SLP-76, SH2 domain-containing leukocyte phosphoprotein of 76 kDa; EVH1, Ena/VASP homology 1; Ena/VASP, enabled/vasodilator-stimulated phosphoprotein; WASP, Wiskott–Aldrich syndrome protein; HIP-55, haematopoetic progenitor kinase 1 interacting protein of 55 kDa; RT, reverse transcription; GST, glutathione *S*-transferase; MYM, multiple yop mutant; ADF-H, actin-depolymerizing factor homology; UMM, ultimate mounting media; Arp, actin related protein; SKAP-55, Src-kinase-associated protein of 55 kDa

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could possibly explain the observed co-localization of Fyb and cellular structures rich in actin dynamics. It was also shown that Fyb became tyrosine-phosphorylated upon TCR stimulation and formed a complex with Wiskott–Aldrich syndrome protein (WASP), VASP, Nck and SLP-76 [18,28]. The role of Fyb in macrophages is largely unknown, but the finding that this adaptor protein is rapidly dephosphorylated by the *Yersinia* antiphagocytic effector, YopH, suggests a role for this adaptor protein in macrophage phagocytosis.

In this paper, we describe the identification of mammalian actin binding protein 1 (mAbp1), also known as SH3p7 or haematopoietic progenitor kinase 1 interacting protein of 55 kDa (HIP-55) [29–31] as a new interaction partner of Fyb. We show that the proline rich N-terminal half of Fyb binds to the SH3 domain of mAbp1 and thus this interaction might be one mechanism through which Fyb influences actin reorganization in macrophages.

2. Materials and methods

2.1. Cell culture

Mouse macrophage-like J774 cells were maintained in α-MEM (Sigma) supplemented with 10% fetal bovine serum (Invitrogen BV), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen) at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Antibodies

HRPO-conjugated anti-phosphotyrosine antibody RC20 (Transduction Laboratories) was used at a dilution of 1:2500 (v/v) for immunoblotting. Mouse monoclonal anti-His antibody (Amersham Bioscience) was used at a dilution of 1:3000 (v/v) for immunoblotting. Rabbit polyclonal anti-Fyb antiserum was kindly provided by Dr. Klemens Rottner (GBF, Germany) and used at a dilution of 1:1000 (v/v) for immunoblotting. GST-mAbp1 1-433 was digested with PreScission protease (Amersham Bioscience) and used to raise polyclonal rabbit antiserum and hen IgY (Agrisera AB, Umeå, Sweden). Both the antiserum and the purified hen IgY recognized both human and mouse mAbp1.

The following secondary antibodies were used: HRPO-conjugated sheep anti-mouse or donkey anti-rabbit antibodies (1:10 000) (Amersham Bioscience, UK), HRPO-conjugated rabbit anti-chicken IgY (1:50 000) (Sigma), FITC-conjugated swine anti-rabbit antibodies (1:100) (Jackson Immuno Research Laboratories Inc.), TRITC- or FITC-conjugated donkey anti-chicken IgY (1:100) (Jackson Immuno Research Laboratories Inc.).

For blocking non-specific staining, 5% normal donkey serum was used (Jackson Immuno Research Laboratories Inc.). For staining of F-actin, 1–2 U/ml phalloidin (Alexa 568 phalloidin; Molecular Probes) was used.

2.3. Construction of GFP-Fyb variants and transfection of J774 cells

Fragments encoding amino acids 1–547, 548–783, 1–783 of Fyb (numbering according to [14,16]) were generated by PCR and cloned into the pCB6 N-GFP vector (kindly provided by Dr. M. Way, EMBL, Heidelberg, Germany [32]). The resulting constructs pCB6GFP-Fyb 1-783 (full length), pCB6GFP-Fyb 1-547, and pCB6GFP-Fyb 548-783 were verified by sequencing. J774 cells were plated onto 12 mm glass cover slips and grown for 1 day before transfection; cells were incubated with a mixture of 3.6 μl of FuGENE 6 reagent (Roche Diagnostics Scandinavia AB) and 0.4 μg of plasmids for 48 h before immunofluorescence assay.

2.4. Yeast two-hybrid assays

Fragments encoding amino acids 1–339, 341–598, 548–783 of Fyb (numbering according to [14,16]) were generated by PCR and cloned into the pGBKT7 (*TRP1* nutritional marker changed to *URA3*) vector (kindly provided by Dr. Anders Byström, Department of Molecular Biology, Umeå University, Sweden) to create a fusion protein with a GAL4 DNA-binding domain. Mouse lymphoma MATCHMAKER cDNA library (BD Biosciences) and the different Fyb constructs were transformed into the yeast strain UMY1974 (*MATa trp1-901 leu2-3, 112 ura3-52 his3-200 gal4 gal80 GAL2-ADE2, LYS2::GAL1-HIS3 met2::GAL7-lacZ*) (kindly provided by Dr. Anders Byström, Department of Molecular Biology, Umeå University, Sweden) by the lithium acetate method described by the manufacturer (BD Biosciences). Positive clones were detected by plating on synthetic medium in the absence of leucine, histidine, uracil and adenine. Plasmids of positive clones were isolated and retransformed to confirm the results and finally verified by sequencing. The β-galactosidase activities of positive clones were measured using the SDS–chloroform method [33].

2.5. Reverse transcription (RT)-PCR

J774 cells mRNA templates were isolated using the Fast Trak kit 2.0 mRNA isolation system (Invitrogen). Primers (5′-CCG GAA TTC CTC TAT GAG CTC CAC GTA GT-3′) and (5′-CGC GGA TCC ATG GCG GTG AAC CTG AGC C-3′) for mAbp1 were used for RT-PCR using the Titan one tube RT-PCR system (Roche Diagnostics Scandinavia AB). The PCR products were run on 1% agarose gel then stained with ethidium bromide. The 1.3 kb band was cut and purified DNA was cloned into pGEX-Teasy vector (Promega) and sequenced.

2.6. GST and his fusion proteins

Fragments encoding amino acids 1–339, 341–598, 548–783 of Fyb were generated by PCR and cloned into the pTrcHisA vector (Invitrogen). Fragments encoding amino acids 1–146, 1–280, 281–433, 371–433, 1–433 of mAbp1 were generated by PCR and cloned into the pGEX-6p-1 vector (Amersham Biosciences). The resulting plasmids were transformed into BL-21 (Stratagene), BL-21 star (DE3) (Invitrogen) or TKX1 (Stratagene). Expression of glutathione *S*-transferase (GST) and His fusion proteins were induced with 0.5 mM of IPTG in the bacteria cultures. Lysates were prepared by sonication. For His fusion proteins, the lysates were separated by SDS–PAGE and transferred onto PVDF membranes (Millipore Corporation), then immunoblotted with anti-pTyr or anti-His antibodies. GST fusion proteins were purified on glutathione Sepharose 4B beads according to the manufacturer's instructions (Amersham Bioscience). The GST fusion proteins were separated by SDS–PAGE then stained with Coomassie blue or transferred onto PVDF membrane and immunoblotted with anti-pTyr antibodies. The GST-mAbp1W/A point mutant, where the tryptophan^{411} was exchanged for alanine, was generated by oligo-based PCR mutagenesis using the QuickChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. All mutations and truncations were verified by sequencing.

2.7. GST pull down

GST-mAbp1 was purified on glutathione Sepharose 4B beads (Amersham Bioscience). For pull down of Fyb from J774 lysates, 10 μg of GST-mAbp1 proteins bound to beads were added to 1 mg of lysates. For pull downs of His-Fyb fusion proteins from bacterial lysates, 5 μg of GST-mAbp1 proteins bound to beads were added to 50 μg of crude lysates in 1 ml of lysis buffer (PBSA, pH 8.0, 1% Triton X-100, 0.2 mM AEBSF) and incubated at 4 °C for 2 h with shaking. The beads were pelleted by centrifugation and washed three times with 1 ml of ice-cold RIPA or lysis buffer, then resuspended in 2 × Laemmlie sample buffer and boiled. The samples were separated by SDS–PAGE, transferred onto PVDF membranes, and immunoblotted [1] with anti-His antibody.

2.8. Bacterial strains and growth conditions

The plasmids encoding wild-type YopH (*pyopH* [34]) and the PTPase inactivated YopH (*pyopH403C/A* [34]) were isolated by standard methods and introduced into the multiple yop mutant (MYM) (*yadA::Tn5*, *yopHMEKypkA*, [35]) strain by electroporation as described previously [20]. The bacteria were grown in Luria Broth (LB) overnight at 26 °C on a rotary shaker. The cultures were then diluted 10× and incubated at 26 °C for 0.5 h, followed by 1 h at 37 °C to induce YopH expression. For infection, the bacteria were pelleted and washed once with prewarmed PBSA then resuspended in prewarmed PBSA to an OD_{600} = 1.0 (about 1×10^9 cfu/ml).

2.9. Infection and immunoprecipitation

J774 cells were seeded in 10 cm tissue culture dishes 1 day before infection to get about 80% confluence. One hour before infection, the medium was exchanged with fresh α-MEM without antibiotic. For infection, the medium was removed and 1 ml of bacteria suspension was added. After incubating for 45 min at 37 °C in a humidified atmosphere with 5% CO_2, the infection was stopped by removing bacteria and washing the cells three times with 10 ml of ice-cold 1× PBSA containing 1 mM Na_3VO_4. The cells were lysed in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, 0.5% Na-SOC, 0.1% SDS and a cocktail of protease inhibitors (Roche Diagnostics Scandinavia AB). Cell lysates were centrifuged for 15 min at 14 000 rpm in a microcentrifuge at 4 °C, cleared cell lysates were collected and protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories). For immunoprecipitation, cell lysates were adjusted for equivalent protein concentration (1 mg) and volume with RIPA buffer, then precleared with 50 μl of 50% protein G Sepharose beads for 1 h at 4 °C. The lysates were subjected to immunoprecipitation with rabbit anti-Fyb (1:50) or anti-mAbp1 (1:100) antisera conjugated to protein G beads for 4 h at 4 °C. After extensive washing in RIPA buffer, immunoprecipitates were subjected to immunoblot analysis with the anti-pTyr antibody RC20, hen anti-mAbp1 antibody and rabbit anti-Fyb antiserum.

2.10. Immunofluorescence staining

J774 cells were plated onto 12 mm glass cover slips and grown for 1 day before staining. The cells were washed, fixed, permeabilized and blocked as described previously [34], then the cover slips were inverted into a drop of PBS (about 40 μl) containing anti-Fyb or anti-SH3p7 antibodies in a humidified chamber and incubated for 1 h. Cover slips were washed in PBS and incubated with 5% donkey normal IgG serum for 20 min, then incubated with secondary antibody for 1 h. All incubations were at 37 °C. The cover slips were finally washed and mounted onto glass slides using ultimate mounting media (UMM) [7]. Samples were analyzed using a fluorescent microscope (Zeiss) with epifluorescent illumination and a 100×/1.4 plan-apochromate oil immersion lens. J774 cells transfected with GFP-Fyb variants were stained for actin using phalloidin, washed and then mounted in UMM [7]. Fluorescent images of transfected cells were obtained with a confocal laser scanning microscope with dual detectors and an argon–krypton (Ar/Kr) laser for simultaneous scanning of the two fluorochromes (Leica). Images of the size 1024 × 1024 were taken using a 63×/1.32–0.6 oil immersion lens with a zoom factor of 5.01 and the pinhole setting was 1.3 airy units.

3. Results

3.1. The Fyb FPPPP motif is not required for localization to actin rich structures

The finding of Fyb as a target of the antiphagocytic bacterial protein, YopH [1], together with data suggesting a role for Fyb in integrin-mediated cell adhesion [23] indicated that this adaptor protein is associated with signalling to the actin cytoskeleton. Fyb, which localizes to actin-rich structures in cells, has previously been shown to interact with the EVH1-domain of Ena/VASP proteins [18], a protein with potential to influence F-actin dynamics [28]. As a first step in the study of the role of Fyb in macrophages and especially in phagocytosis, the importance of the Fyb-Ena/VASP interaction for localization of Fyb to cellular actin rich structures was investigated. J774 cells were transfected with GFP-Fyb full length 1–783, GFP-Fyb 1–547, which lacks the EVH1-binding FPPPP motif, GFP-Fyb 548–783, and GFP alone. Microscope analyses of

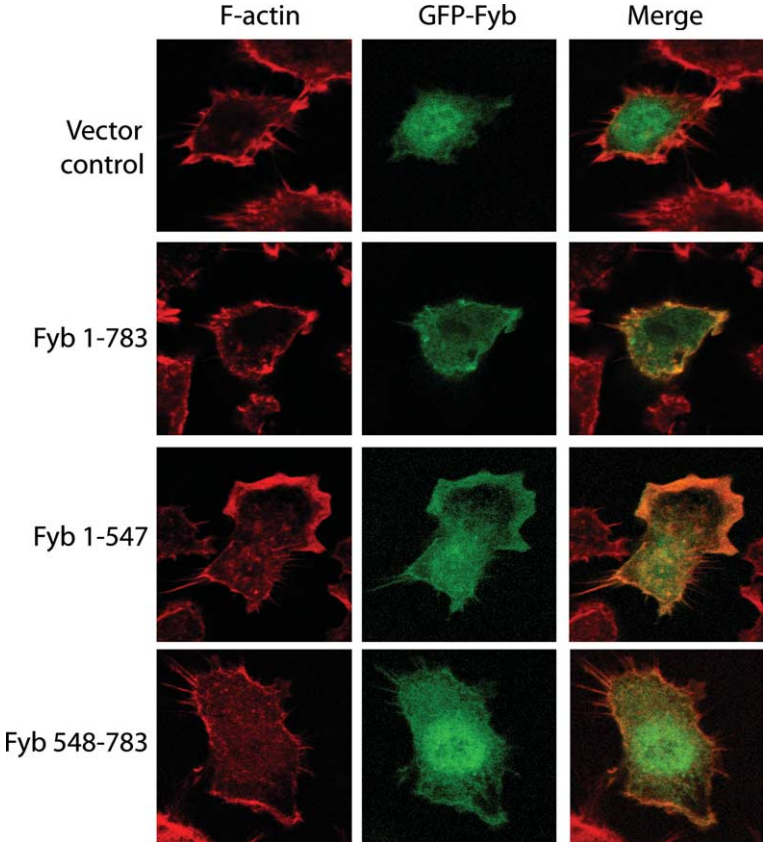

Fig. 1. Co-localization of Fyb and F-actin. J774 cells transfected with pCB6GFP vector as a control, pCB6GFP-Fyb 1–783 (full-length Fyb), pCB6GFP-Fyb 1–547 or pCB6GFP-Fyb 548–783 were fixed and stained with phalloidin (red). The specimens were examined by confocal immunofluorescence microscopy.

the transfected cells revealed that all constructs except the GFP control localized to actin-rich lamellipodia (Fig. 1). This indicated that the FPPPP motif not is a prerequisite for the association of Fyb with F-actin rich structures in cells, and that also an additional part of the Fyb protein directly or indirectly can mediate this interaction. Moreover, pull down experiments using GST-Fyb 548–783 revealed that Fyb interacted with a minor portion of total VASP in J774 lysates (supplementary data Fig. 1).

3.2. N-terminal Fyb interacts with mAbp1 in the yeast two-hybrid system

Fyb contains several functional domains that can mediate protein–protein interactions. In an attempt to identify novel Fyb binding proteins, truncated variants of Fyb encompassing amino acids 1–339, 341–598, and 548–783, respectively, were used as bait to screen a mouse lymphoma cDNA library using the yeast two-hybrid system. With Fyb 1–339 as bait, we found a positive clone that represented a 1.2 kb cDNA insert encoding mouse mAbp1 149–433 amino acids. Co-transformation of the mAbp1 cDNA with the three Fyb fragments revealed that yeast cells containing mAbp1 together with Fyb 1–339, but not Fyb 341–598 and 546–783 grew on plates lacking leucine, histidine, uracil and adenine; the empty vector pGBKT7 was used as control (Fig. 2A). Similar results were obtained by the β-galactosidase activity assay (Fig. 2B).

3.3. MAbp1 is expressed in J774 cells and localizes together with Fyb in F-actin rich areas

Mouse mAbp1 is a ubiquitously expressed protein, 433 amino acids long, which can bind F-actin and it has been implicated in regulation of endocytosis [36,37]. Since Fyb has been identified as the YopH substrate in J774 macrophages, these cells were chosen for further studies of the Fyb-mAbp1 interaction. Initially, RT-PCR of mRNA from J774 cells was employed to investigate whether mAbp1 was expressed in these cells. RT-PCR with primers for mouse mAbp1 resulted in a single band about 1.3 kb (Fig. 3A). Sequencing of this fragment confirmed that the band corresponded to full-length mouse mAbp1. The DNA fragment was further later cloned into pGEX-Teasy vector. Purified GST-mAbp1 protein was thereafter used to raise antibodies against mAbp1.

Immunofluorescence staining of J774 cells showed that mAbp1 localized in the perinuclear region and in the leading edge where it co-localized with F-actin (Fig. 3B), a localization that corresponds to that previously shown for Fyb in other cell types [23]. Co-staining of Fyb and mAbp1 showed that these proteins co-localized in areas with high actin dynamics, cell edges and ruffles (Fig. 3C). The colocalization of Fyb and mAbp1 was strictly observed at the cell edges and neither were any of the proteins found in punctuate structures, resembling adhesion structures. The latter is in agreement with previous data shown that Fyb not localizes to adhesion structures [18].

3.4. MAbp1 binds Fyb in J774 cells

To determine if mAbp1 interacts with Fyb in macrophages, GST-mAbp1 protein was purified and used in pull down experiments with J774 cell lysates. The precipitated proteins were separated on SDS–PAGE, transferred onto a PVDF membrane and blotted with anti-Fyb antibodies. The anti-Fyb antibody recognized two bands about 120–130 kDa in the J774 lysate, which correspond to the two isoforms of Fyb. These bands were also detected in the sample of GST-mAbp1 pull down, but not in that with GST alone, indicating

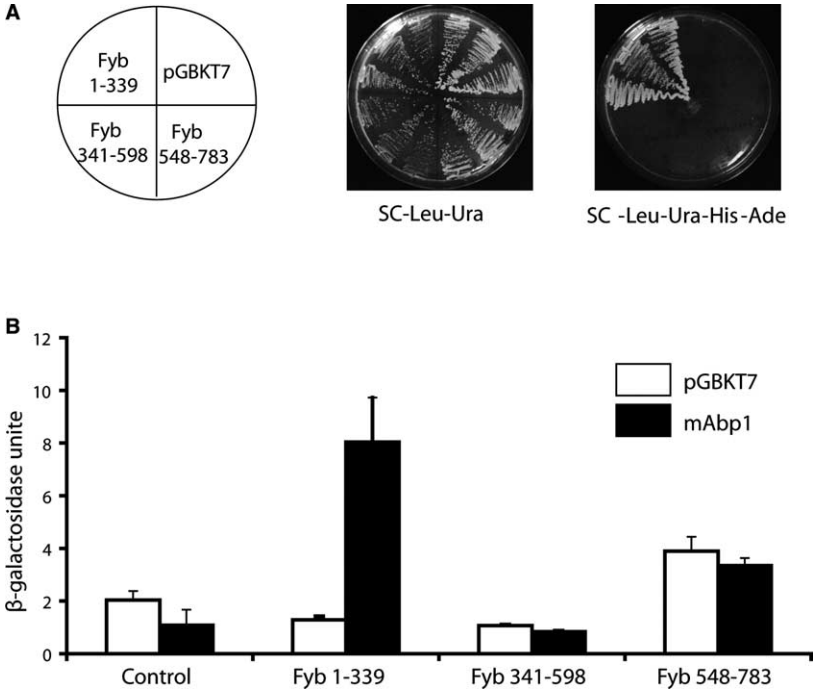

Fig. 2. Interaction of Fyb with mAbp1 in the yeast two-hybrid system. Fyb truncated domains (1–339, 341–598, 548–783) in pGBKT7 vectors and empty pGBKT7 were transformed together with mAbp 149–433 in pACTII vector (positive clone of mouse lymphoma MATCHMARK cDNA library screen) into the yeast strain UMY1974. (A) Growth on selective plates. Three single transformants of each transformation were spread on SC-Leu-Ura-His-Ade and SC-Leu-Ura plates and incubated at 30 °C. (B) β-Galactosidase assay; β-Galactosidase activity in three transformants of each transformation were measured using the SDS–chloroform method.

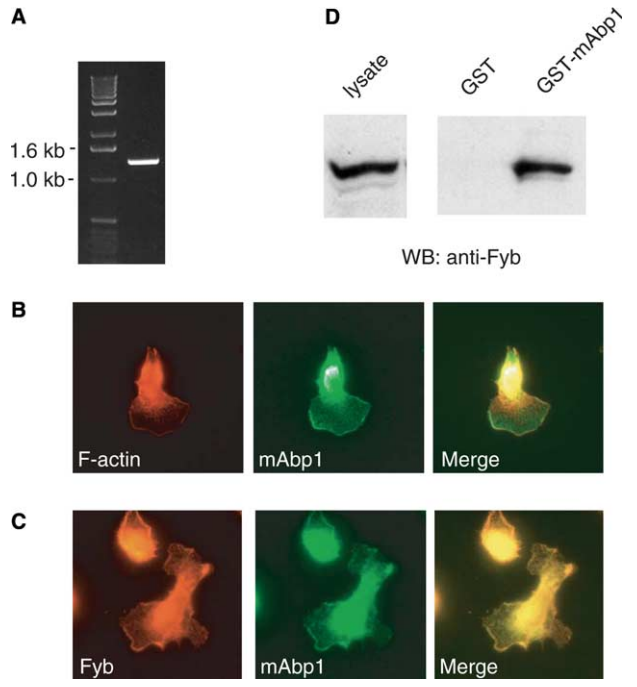

Fig. 3. Interaction of Fyb with mAbp1 in mouse macrophage-like J774 cells. (A) RT-PCR of mAbp1; J774 cell mRNA was purified and subjected to RT-PCR with mAbp1 primers. The PCR products were separated on 1% agarose gel. The molecular weight marker is indicated on the left. (B) Co-localization of mAbp1 and F-actin; J774 cells were fixed and stained with hen anti-mAbp1 antibody (green) or phalloidin (red). The specimens were examined in a fluorescence microscope. (C) Co-localization of Fyb and mAbp1 in J774 cells; J774 cells were fixed and stained with hen anti-mAbp1 antibody (red) and rabbit anti-Fyb antiserum (green). The specimens were examined in a fluorescence microscope. (D) GST-mAbp1 binds Fyb in J774 cell lysates; GST-mAbp1 on beads was used to pull down Fyb from J774 cell lysates, GST beads were used as a control. The proteins bound to the beads were separated on SDS–PAGE gel and transferred onto PVDF membrane, then probed with anti-Fyb antiserum. 10% lysates from pull down experiments were loaded in the left lane.

that mAbp1 can interact with endogenous Fyb in macrophages (Fig. 3D). Loading various concentrations of cell lysates revealed that about 10% of total Fyb was pulled down with GST-mAbp1 (data not shown).

3.5. Fyb interacts with the mAbp1 SH3 domain

The mAbp1 protein is an actin binding protein, which has a single actin-depolymerizing factor homology (ADF-H) domain at its N-terminus, followed by an α-helical structure region, a stretch with PxxP and YXXP motifs and a C-terminal SH3 domain (Fig. 4B) [29–31]. To investigate which part of mAbp1 was responsible for the interaction with Fyb, different variants of GST-mAbp1 and His-Fyb protein fragments were generated for use in in vitro interaction studies (Fig. 4A and B). For mAbp1, pGEX-mAbp1 encoding full-length mAbp1, pGEX-mAbp1 1–146 encoding the ADF-H domain alone, pGEX-mAbp1 1–280 encoding the ADF-H domain and the α-helical region, pGEX-mAbp1 281–433 encoding the C-terminal half with the PxxP and YxxP regions and the SH3 domain, and pGEX-mAbp1 371–433 with the SH3 domain alone were expressed in BL-21 and purified on glutathione Sepharose 4B beads. Coomassie staining of the separated proteins revealed that all variants were expressed and had the estimated molecular weight (Fig. 4D). Similarly, constructs encoding His-tagged Fyb protein fragments encompassing residues 1–339, 341–598 or 548–783 were generated and expressed in BL-21. The bacterial lysates were subjected to Western blotting using anti-His antibodies to confirm expression (Fig. 4C). Both Fyb and mAbp1 contain tyrosine motifs, and to investigate if any tyrosine-phosphorylated motif is involved in the interaction, tyrosine phosphorylated variants of the mAbp1 and Fyb proteins were also generated. For this, the proteins were expressed in TKX1, which carries an inducible tyrosine kinase gene [38]. Western blotting using anti-phosphotyrosine antibodies of proteins generated by TKX1 showed that all GST-mAbp1variants and His-Fyb 341–598 as well as His-Fyb 548–783 were tyrosine phosphorylated (data not shown). The same molecular amounts of mAbp1 GST fusion proteins on beads were used in pull down experiments with bacterial lysates containing equal amounts of the different His-Fyb proteins. His-Fyb proteins that bound to mAbp1-beads were detected with anti-His antibodies. In accordance with the results of the yeast two-hybrid experiment, full-length mAbp1 bound to Fyb 1–339, but not to Fyb 341–598 or Fyb 548–783 (Fig. 4E). In addition, both tyrosine-phosphorylated and non-tyrosine phosphorylated mAbp1 bound to the Fyb 1–339 fragment (Fig. 4E).

To investigate which part of the mAbp1 protein was involved in the interaction with Fyb, GST-mAbp1 protein variants were used in pull down experiments with His-Fyb 1–339 lysates. Here, the two C-terminal fragments mAbp1 281–433 and mAbp1 371–433, which both encompass the SH3-like domain, interacted with Fyb (Fig. 4F). Also here, tyrosine phosphorylation of the proteins was not necessary for the interaction (Fig. 4F). No interaction was seen using the other SH3-domain deficient mAbp1 variants. Hence, the N-terminal part of Fyb, which contains several proline-rich regions, binds to the mAbp1 SH3 domain. To further confirm that the interaction between mAbp1 and Fyb was mediated by a typical SH3 domain interaction, an SH3-dominant negative mAbp1 point mutant was constructed. In this mutant, GST-mAbp1W/A, the conserved tryptophan in the SH3 domain [39], had been exchanged with an alanine. Pull down experiments showed that GST-mAbp1W/A failed to interact with His-Fyb 1–339 (Fig. 4G), further indicating that mAbp1 interacted with Fyb through SH3-proline binding.

3.6. Fyb interacts with mAbp1 in vivo and both are tyrosine-dephosphorylated by YopH in infected J774 cells

Prior studies have shown that the *Yersinia* antiphagocytic factor, YopH, targets tyrosine-phosphorylated Fyb proteins in J774 cells. To test whether the Fyb interacting protein, mAbp1, is also dephosphorylated by YopH, and if bacterial infections affect the Fyb-mAbp1 interaction, J774 cells were infected with variants of the *Yersinia pseudotuberculosis* MYM strain before analysis. The MYM strain is defective in the YadA, YopM, E, K, H, and YpkA virulence effectors, but the genes encoding proteins necessary for regulation, secretion and translocation are intact [35]. In this study, we used MYM alone, MYM expressing the PTPase active wild-type YopH (MYMYopH), and MYM expressing a catalytically inactive YopH (MYMYopH403C/A) [1,34]. The J774 cells were left uninfected or infected with the different MYM strains for 45 min. Fyb or mAbp1 antisera were thereafter used for immunoprecipitation of the cell lysates. The precipitated proteins

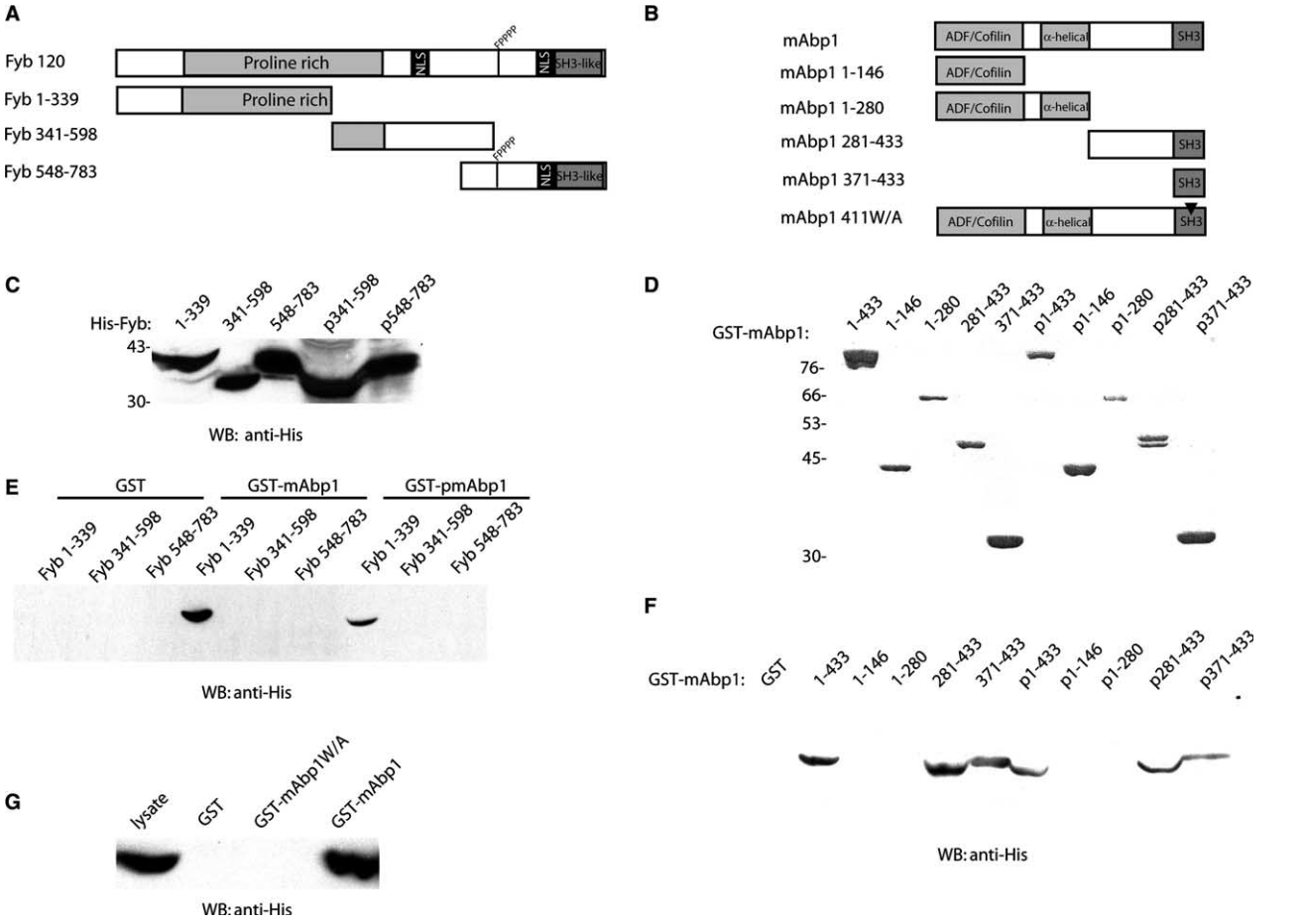

Fig. 4. The N-terminal part of Fyb binds to the mAbp1 SH3 domain. (A) Schematic representation of the His-Fyb proteins used in the study. The proline-rich (P) region (residues 100–440), the putative nuclear localization sequence (NLS) (residues 468–505, 674–700) and the SH3-like domain (residues 371–433) are indicated. For simplicity, the N-terminal His-tags are omitted. (B) Schematic representation of the GST-mAbp1 proteins used in the study. The presentation of the ADF-H/cofilin region (residues 1–140), the α-helical structure (residues 141–280) and the SH3 domain (residues 371–433) are indicated. For simplicity, the N-terminal GSTs are omitted. (C) Expression of His-Fyb fusion proteins. Crude lysates of His fusion proteins were subjected to SDS–PAGE and transferred to PVDF membrane then blotted with anti-His antibodies. The molecular weight markers are indicated on the left. (D) Expression of GST-mAbp1 fusion proteins; Purified GST fusion proteins were subjected to SDS–PAGE and stained with Coomassie Blue. The molecular weight markers are indicated on the left. (E) N-terminal Fyb interacts with mAbp1; GST-mAbp1 and GST-pmAbp1 were used to pull down His-Fyb 1–339; GST beads were included as control. The proteins bound to beads were detected with anti-His antibodies. (F) Fyb interacts with the SH3 domain of mAbp1; Full length GST-mAbp1 and the different truncated variants (indicated above each lane) were used to pull down His-Fyb 1–339 in vitro. The proteins bound to beads were detected with anti-His antibodies. (G) SH3 mutant of mAbp1 fails to interact with Fyb; GST-mAbp1 W/A mutant was used to pull down His-Fyb 1–339 in vitro, GST and GST-mAbp1 were used as controls. The proteins bound to the beads were detected with anti-His antibodies.

were subjected to SDS–PAGE, transferred to PVDF membranes, and blotted with anti-pTyr, anti-Fyb or anti-mAbp1 antibodies. The results showed that both Fyb and mAbp1 in the J774 cells were present in tyrosine-phosphorylated forms, and that the bacterial infection caused an increased phosphorylation of Fyb, but not of mAbp1 (Fig. 5A and B). Moreover, YopH dephosphorylated both proteins (Fig. 5A and B), implicating that mAbp1 might also be a substrate for this effector. In addition, in accordance with the pull down results, mAbp1 was co-immunoprecipitated with Fyb (Fig. 5C). Noteworthy, there were no differences between the Fyb-mAbp1 interactions detected in non-infected cells compared to cells infected with MYM expressing the catalytically active YopH (Fig. 5C). In accordance, co-staining of Fyb and mAbp1 in J774 cells infected with this strain, showed that these two proteins were co-localized at the cell edges also in the presence of active YopH (supplementary data Fig. 2). Hence, Fyb-mAbp1 interaction is not interrupted by YopH, which is in agreement with the observed phosphotyrosine independence of this interaction.

4. Discussion

Here we show, for the first time, that the immune cell specific adapter protein, Fyb, interacts with the F-actin-binding protein, mAbp1, in mouse macrophage cells. We have studied the interaction in detail and shown that it is the C-terminal SH3 domain of mAbp1 that interacts with the N-terminal half of Fyb. The role and function of Fyb in macrophages has been obscure, although the identification of this adapter as a substrate of the bacterial antiphagocytic factor, YopH, suggested

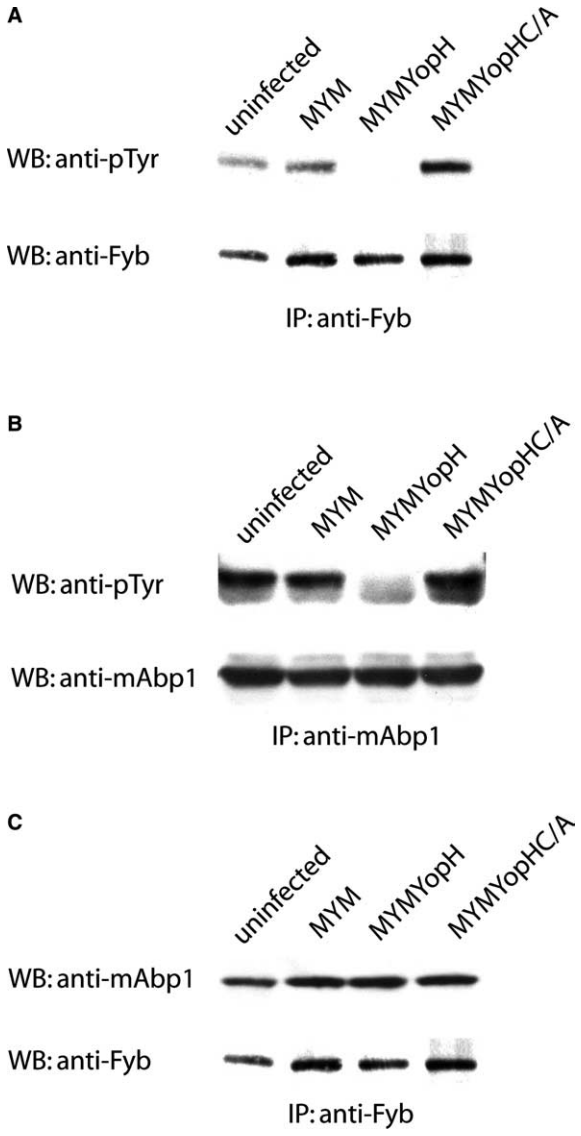

Fig. 5. YopH dephosphorylates the Fyb–mAbp1 complex in J774 cells. J774 cells were left uninfected or infected with MYM, MYMYopH or MYMYopHC/A for 45 min. The calculated bacteria/cell ratio was 100:1. The infected cells were lysed, the cell lysates were subjected to immunoprecipitation with rabbit anti-Fyb (A, C) or anti-mAbp1 (B) antisera and subsequently immunoblotted with the indicated antibodies.

a role in phagocytosis. Fyb has previously been implicated to influence actin dynamics and integrin clustering in cells, but the mechanism behind this has remained unknown. Consequently, the present finding that Fyb can bind mAbp1 further supports a role for Fyb in signalling for F-actin dynamics and also provides additional insight into the mechanism involved.

The mAbp1 protein is a ubiquitously expressed F-actin binding protein [29,31,36]. It localizes to the perinuclear region of cells and is specifically recruited to newly forming F-actin structures during signalling leading to Rac activation, such as lamellipodia in moving and spreading cells [36]. We show that Fyb interacts with mAbp1 both in vivo and in vitro, and that this protein complex is present in the cellular perinuclear region and enriched in lamellipodia structures that are directed forward. In addition to the F-actin binding properties, mAbp1 can, via its SH3 domain, interact directly with dynamin, the GTPase that regulates fission of endocytic vesicles [29]. Noteworthy here is that over-expression of the dynamin-binding SH3 domain has been shown to cause blockage in endocytosis [29]. Other proteins known to interact with the mAbp1 SH3 domain are haematopoietic progenitor kinase 1, the presynaptic zinc finger protein, piccolo, and the Cdc42 GEF, Fgd1 [30,40,41]. The latter two binding partners have clear connections to actin dynamics: Fgd1 can stimulate F-actin reorganization via the Rho GTPase, Cdc42 [42], and piccolo has been suggested to act as a scaffold protein that can bind both profilin and actin and is involved in synaptic vesicle endo- and exocytosis near their site of action [40]. Given these dual properties, it is anticipated that mAbp1 plays a role in the coordination of endocytic and cytoskeletal activities.

The N-terminal F-actin binding ADH/cofilin homology domain, and the observed localization to F-actin rich structures suggest that mAbp1 participate in the regulation of actin dynamics. The yeast orthologue of mAbp1, which has also been implicated in endocytic processes, has been found to be an activator of the actin related protein (Arp) 2/3 complex and is critical in regulating the actin cytoskeleton and cell polarity in these cells [43,44]. A direct activation of Arp 2/3 by mAbp1 has not been reported. Although mAbp1 co-localizes with Arp 2/3 in the leading edges of newly formed lamellipodia [36], the acid regions in the yeast Abp1 ADH/cofilin domain, shown to be required for Arp 2/3 activating activity, are, however, not present in mAbp1. RNAi of mAbp1 in human embryonic kidney cells has revealed that this protein is essential for endocytosis but not for lamellipodia formation [37]. However, this does not rule out a role for mAbp1 in directly regulating F-actin; even if F-actin dynamics leading to membrane extensions is less likely, mAbp1 can be important for F-actin dependent events involved in membrane invagination and vesicle trafficking. Both these events are essential steps in phagocytosis, and we find it intriguing that Fyb, which is a target for an antiphagocytic factor in macrophages, binds to a protein with potential to regulate F-actin-dependent vesicle uptake. We also observed that upon infection of macrophages with *Yersinia*, the antiphagocytic factor, the PTPase YopH, dephosphorylated both Fyb and mAbp1. However, this did not affect the Fyb-mAbp1 interaction, and we speculate that dephosphorylation of Fyb is sufficient to impair the function of the complex. It has been shown previously that mAbp1, which is tyrosine-phosphorylated by Src kinases, localizes to areas with newly formed actin structures independent of tyrosine phosphorylation [36]. Our observation that the β1-integrin engagement caused by the bacterial infection stimulates tyrosine phosphorylation of Fyb but not of mAbp1, supports a model where Fyb tyrosine phosphorylation is involved in the receptor-mediated engagement of this protein, and that mAbp1 tyrosine phosphorylation is less important. This does not exclude a role for mAbp1 in the phagocytic process; its potential properties to coordinate F-actin and endocytosis rather point towards a role in the phagocytic uptake and work to reveal the mechanism of mAbp1 in this process is therefore ongoing.

Fyb has been shown to bind many different molecules, such as the tyrosine kinase Fyn [16], Ena/VASP family proteins [18], SLP-76 [16], and Src-kinase-associated protein of 55 kDa (SKAP-55) [45]. Interestingly, a complex of Fyb, Nck, VASP and WASP, which is an activator of Arp 2/3 [46] has been found to translocate to phagocytic cups upon Fcγ receptor-

mediated phagocytosis [27]. WASP is an activator of Arp 2/3 and is speculated to contribute to local actin rearrangements at this site. Fyb does not bind directly to Nck or WASP, but it is likely that these are recruited via the SLP-76 protein that binds Nck and Vav, a guanine exchange factor that promotes activation of Rho GTPases of which WASP is a downstream effector. Accordingly, our finding of mAbp1 as an interactor of Fyb suggests a possible linker of the WASP-containing complex to the F-actin network.

Moreover, it was recently reported that mAbp1 participates in signalling from the immunologic synapse, a macromolecular structure at the T cell-APC interface. The mAbp1 protein was suggested to modulate T cell activation by connecting T cell receptors to actin cytoskeleton and endocytic processes [47]. Interestingly, Fyb has also been implicated in signalling from the T cell receptor, where it is recruited to the forming immunological synapse, and in analogy with that seen for mAbp1, there is no requirement for Fyb in formation and T cell-APC conjugates or actin polymerization [18,48]. In the case of Fyb, the effects were on signalling from the T cell receptor to the integrins [24,25]. Although the role of mAbp1 in the coupling of T cell receptor-mediated actin cytoskeletal rearrangement with activation of integrin function remains to be elucidated, there are common features that suggest that Fyb and mAbp1 participate in similar events in T cells.

The present study shows that the immune cell specific adapter protein Fyb interacts with mAbp1, a protein that can influence cytoskeletal and endocytic events, and suggests a potential mechanism by which Fyb can influence F-actin reorganization.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2005.03.031.

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